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The role of bacterial phytotoxins in inhibiting the eukaryotic proteasome

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Abstract

The ubiquitin-26S proteasome degradation system (UPS) plays a pivotal role in almost all aspects of plant life, including defending against pathogens. While the proteasome is important for plant immunity, it has been found to also be exploited by pathogens using effectors to increase their virulence. Recent work on the XopJ effector and syringolin A/syrbactins has highlighted host proteasome inhibition as a virulence strategy of pathogens. This review will focus on these recent developments.

The ubiquitin-26S proteasome degradation system (UPS) and plant immunity

Besides the lysosome, the UPS is the main protein degradation system of eukaryotic cells that not only destructs misfolded proteins but is also involved in many cellular processes by degrading proteins regulating such processes [1, 2]. Proteins destined for destruction by the proteasome become polyubiquitinated by a reaction cascade involving three enzymes designated E1, E2, and E3. Ubiquitin is activated in an ATP-dependent way by E1 and transferred to E2, from which it is normally directly transferred to a lysine residue of the target protein by E3, which, by selecting target proteins, confers specificity to the UPS [3]. Polyubiquitination is achieved by multiple rounds in which further ubiquitin units are conjugated to an internal lysine residue of ubiquitin. Polyubiquitinated proteins are substrates for the proteasome, which is composed of two 19S regulatory particles (RP) capping a 20S core particle (CP). The RPs recognize polyubiquitinated proteins, which become deubiquitinated and unfolded in an ATP-dependent reaction, and regulate access of unfolded substrates to the proteolytic channel of the CP. The channel is formed by four stacked seven-membered rings. The two identical outer rings consist of seven different α-subunits, whereas

the two identical inner rings consist of seven different β-subunits, three of which have proteolytic activities: the β1 subunit exhibits a caspase-like activity (CL), cleaving protein substrates after acidic residues, while the β2 and β5 subunits have trypsin-like (TL) and chymotrypsin-like (ChTL) activities cleaving after basic and hydrophobic residues, respectively. In all three catalytic subunits, the active site residue is the N-terminal threonine (Thr) [4].

The UPS is involved in the signaling pathways of nearly all plant hormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), hormones of particular importance for plant defense against pathogens [5]. SA plays a prominent role in defense reactions directed mainly against biotrophic pathogens [6]. Many of the SA-dependent defense reactions, such as *PR* (*PATHOGENESIS-RELATED*) gene activation, require the key regulator NPR1 (NONEXPRESSOR OF PR GENES 1), which must be turned over by the UPS to function properly [7]. In contrast, JA and ET orchestrate defense reactions mainly directed against necrotrophic pathogens [6]. The JA receptor is part of an E3 ligase targeting JAZ (JA ZIM domain) proteins for proteasomal destruction. The destruction of JAZ proteins releases transcription factors such as MYC2 from sequestration by JAZ proteins and thus leads to the activation of JA response genes [8]. The UPS is also required at multiple steps in ET signaling, such as the regulation of ET biosynthesis and downstream signaling components. In addition to these three well-known defense hormones, other hormones such as auxin, gibberellic acid, and abscisic acid, which all require the UPS in their signal transduction pathways, have also been shown to affect the interactions of plants with pathogens [9]. Apart from hormone signaling, a considerable number of E3 ligases and other UPS components have been found to play a role in plant immunity, further testifying to the importance of the UPS in pathogen defense [10].

Exploitation of the UPS by pathogens

Not only is the UPS involved in many aspects of plant immunity, but a variety of plant and animal pathogens subvert the host UPS for their own good [11]. To see how, it may be helpful to shortly summarize the major paradigms of plant defense against pathogens. As worked out mainly in the model plant *Arabidopsis thaliana,* plants have evolved a two-branched innate immune system to combat pathogens [12]. One branch is named pattern-triggered immunity (PTI) and involves a plethora of physiological and cellular defense responses adverse to pathogens, conferring basal resistance. PTI is launched after recognition of pathogens (or microbe)-associated molecular patterns (PAMPs or MAMPs) by membrane-bound pattern recognition receptors (PRRs), which exhibit structural similarity to the Toll-like innate immune receptors in mammals and insects [13, 14]. Bacterial pathogens are able to suppress PTI by using their type III secretion system (T3SS) to inject effector proteins (T3Es) into the cytoplasm of host plant cells where they block PTI-associated signaling at many points, thus resulting in effector-triggered susceptibility (ETS) [15, 16]. However, plants have evolved a second branch of innate immunity dubbed effector-triggered immunity (ETI), in which specific effectors are recognized by cognate resistance (R) proteins. This usually results in a hypersensitive reaction (HR), a form of local programmed cell death conferring nearly complete but race-specific resistance [17]. In many plants, local HR induces systemic acquired resistance against a broad spectrum of pathogens in remote tissues [18]. T3Es are known to interfere with all these defense reactions at various points [15, 16].

Analyses of several plant–pathogen interaction systems have identified a number of T3Es that require the host proteasome for their defense-suppressing activities, thus subverting it for their own use [11]. A well-studied example is HopAB2 (alias AvrPtoB) produced by the tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and some other strains that exhibits E3 ligase activity and targets host defense proteins such as PRRs for proteasomal destruction [19-23]. Recently, XopL, a T3E from the pepper pathogen *Xanthomonas*

campestris pv. *vesicatoria* suppressing cell death and PTI, also was shown to have E3 ligase activity and to exhibit a novel architecture [24].

Another example is HopM1 produced by *Pst* DC3000 ($HopM1_{DC3000}$), which mediates the proteasomal degradation of AtMIN7 (*A. thaliana* HopM1 Interactor 7), a guanine nucleotide exchange factor required for proper callose deposition, a PTI response [25]. At present it is not clear whether $HopM1_{DC3000}$ is an E3 ligase itself or whether it recruits an unknown E3 ligase [25]. Furthermore, a T3E family of plant-type F-box proteins (named GALAs) is encoded in the genome of *Ralstonia solanacearum* strains [26], whose members are thought to be part of SCF-type E3 ligases targeting as yet unknown host proteins for destruction [27, 28]. GALA7 is necessary for colonization of *Medicago truncatula*, and deletion of all seven *GALA* genes of *R. solanacearum* strain GMI1000 led to loss of pathogenicity on *A. thaliana* and reduced virulence on tomato [28]. In addition, F-box domain-containing proteins have been identified in *Mesorhizobium loti, Xanthomonas campestris* pv. *vesicatoria*, and *P. syringae* pv. *phaseolicola*. T3Es exhibiting E3 ligase activity have also been identified in bacteria pathogenic to animals [11], such as the NleG and NleL proteins from enterohaemorrhagic *Escherichia coli* [29, 30], the IpaH proteins of *Shigella flexneri* [31, 32], as well as SspH2 and SopA of *Salmonella* [33, 34]. Exploitation of the host proteasome for the enhancement of virulence may be quite widespread not only in pathogenic bacteria, but also in other pathogens such as the oomycete *Phytophthora infestans* which produces the RXLR motif-containing effector AVR3. AVR3 is translocated into the cytoplasm of host cells where it stabilizes the E3 ligase CMPG1, thereby suppressing CMPG1-dependent cell death triggered by pathogen recognition [35, 36].

The examples briefly discussed above show that exploitation of the host proteasome function is employed by a wide variety of pathogens to enhance their virulence. In contrast, inhibition of the host proteasome has only recently been recognized as a virulence strategy of

certain pathogens. The rest of the review focuses on this recent development and covers important new research, extending a recent review with a similar topic [37].

Proteasome inhibition as a virulence strategy of plant pathogens

A T3E with proteasome inhibiting activity

An intriguing case of proteasome inhibition to enhance virulence has recently been reported by Üstün and colleagues [38], who identified the first T3E effector that suppresses proteasome activity. The *X. campestris* T3E XopJ was shown to interact with the RPT6 (regulatory particle ATPase 6) subunit of the 19S RP in yeast and *in planta* and to inhibit the proteasome. XopJ belongs to the YopJ T3E family of cysteine proteases/acyltransferases that is widely distributed in plant and animal pathogens as well as in plant symbionts and exhibits a characteristic catalytic triad [39]. Mutation of the catalytic triad as well as of the N-terminal myristoylation motif in XopJ abolished its proteasome-inhibiting ability, suggesting that enzymatic activity and membrane anchoring are required for proteasome inhibition. Comparisons of wild-type and mutant strains revealed that XopJ delayed host tissue degeneration and suppressed the accumulation of normal levels of SA and SA-dependent defense responses. It is presently not clear how this works mechanistically in view of the necessary localization of XopJ at the membrane. Future research will without doubt elucidate whether proteasome inhibition is the mode of action also of other members of the YopJ superfamily of T3Es. The study of Üstün and co-workers will likely also stimulate the search for T3E-type proteasome inhibitors outside the YopJ family of proteins in other pathogens.

Syringolin A, a small-molecule proteasome inhibitor and virulence factor

Many potent small-molecule proteasome inhibitors belonging to different structural classes have been isolated from microbes and other organisms such as sponges and bryozoans (in which they probably are produced by symbiotic bacteria). While they draw intense attention as potential drug leads in the fight against certain cancer types [40], the function of these compounds for the biology of their producers remains in most cases completely unexplored. An exception is syringolin A (SylA), which is discussed below.

SylA was found to be secreted by strains of *P. syringae* pv. *syringae* (*Pss*). SylA is a tripeptide derivative consisting of an N-terminal valine and the two non-proteinogenic amino acids 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid which form a twelve-membered macrolactam ring. The N-terminal valine is joined head-to-head to another valine residue via an unusual ureido group (Figure 1). SylA is the major variant of a family of related compounds in which the valine residues can be substituted by isoleucine and/or the 3,4-dehydrolysine may be replaced by lysine [37, 41-43]. SylA was shown to covalently attach to the catalytic N-terminal Thr residue of all catalytic subunits of the proteasome by a Michael-type 1,4-addition to the double bond at C4 in the 12-membered macrolactam ring, thereby irreversibly inhibiting catalytic activity [44]. Proteasome inhibition is probably specific because cysteine proteases and trypsin were not inhibited. Although all three catalytic activities of the proteasome are inhibited in vitro, the β5 subunit with its ChTL activity is most sensitive, while the β1 subunit conferring CL activity is least sensitive to inhibition by SylA [44]. Similar results were obtained in living plant cells by proteasome activity imaging and profiling experiments, in which SylA seemed to target preferentially proteasomes in the nucleus [45]. As recently elucidated in *A. thaliana*, SylA is efficiently taken up into plant cells by YELLOW STRIPE LIKE7 (YSL7) and YSL8 transporters [46], which belong to a plant-specific oligopeptide transporter subfamily thought to be involved in transport and distribution of metal-nicotianamine complexes [47]. SylA is the founding member of a new

structural class of proteasome inhibitors dubbed syrbactins, which is characterized by the 12-membered macrolactam ring and its functional α,β-unsaturated carbonyl group [48, 49].

Spray-inoculation experiments with the SylA-producing wild-type strain *Pss* B728a and a SylA-negative mutant on their bean (*Phaseolus vulgaris*) host revealed the mutant to cause only about 30% of brown spot disease symptoms as compared to the wild type, identifying SylA as a virulence factor [44]. Further experiments revealed that SylA not only counteracted stomatal immunity (i.e. the pathogen-induced closure of the stomata, a PTI response dependent on the SA defense pathway and its key regulator NPR1[50-52]) in bean and *Arabidopsis*, but also inhibited other SA-dependent defense reactions such as *PR* gene induction [52]. Inhibition of the SA defense pathway by SylA is most likely due to inhibition of the proteasome-mediated turnover of the key regulator NPR1, which is necessary for its proper functioning as mentioned above. Furthermore, wound inoculation experiments with SylA-producing *Pss* strains on *Nicotiana benthamiana* recently revealed that SylA-mediated suppression of the SA defense pathway also helps the bacteria to escape local wound sites and to colonize adjacent regions along the vascular tissue because acquired resistance was impaired [53].

Occurrence of SylA synthetase genes in *P. syringae* strains

The SylA synthetase, a modular mixed non-ribosomal peptide synthetase/polyketide synthase (NRPS/PKS; see Box 1) is encoded by a gene cluster containing the five genes *sylA, sylB, sylC, sylD,* and *sylE*, the last three forming an operon [54, 55] (Figure 2A). Sequence and architecture of these genes implied a SylA biosynthesis model also experimentally supported in most of its aspects [54-58] (Figure 2B). Importantly, the structure of SylA with its 12-membered macrolactam ring containing the functional α,β-unsaturated carbonyl group is reflected in the unique architecture of the *sylD* gene. This allows the identification in genome

data bases of homologous genes likely encoding structurally similar proteasome inhibitors in other taxa.

A recent genomics analysis of 19 diverse strains belonging to the *P. syringae* species complex revealed complete *syl* gene clusters with very high sequence conservation at conserved positions in the genomes of five of six strains belonging to group (subclade) 2, but not in strains belonging to groups 1 and 3, suggesting that SylA biosynthesis may be restricted to group 2 strains (Figure 3) [59]. Interestingly, group 2 strains seem to encode fewer T3Es (9 to 16) than strains of the other groups. The comparatively low numbers of T3Es encoded in the analyzed group 2 strains, which correlates with the presence genes for the biosynthesis of the phytotoxins syringopeptin and syringomycin as well as of syringolin, suggests a virulence strategy distinct from the one of group 1 (which encompasses *Pst* DC3000) and group 3 strains [59].

Conflicting action of virulence factors?

Concomitant production of proteasome inhibitors with virulence factors requiring the host proteasome for their activity seems contradictory. In *X. campestris* pv. *vesicatoria*, the T3Es XopJ (proteasome inhibitor) and XopL (an E3 ligase) represent such a pair. The seeming conflict might be mitigated by spacial and/or temporal separation of production or activities [38]. In *P. syringae,* SylA production would seem to counteract host proteasome-requiring T3Es like HopAB2 and HopM1 or other virulence factors such as the JA- mimicking small molecule coronatine (COR). To our knowledge, COR synthetase genes or COR biosynthesis so far have not been found in strains carrying SylA synthetase genes [59, 60]. A homolog of HopAB2 (HopAB1, alias $AvrPtoB_{B728a}$) occurs in the SylA-producing strain *Pss* B728a, which, however, has recently been shown to be defect in its E3 ligase activity [61]. In the case of HopM1, a variant of which occurs in *Pss* B728a as well as in most *P. syringae* strains, it is

also not clear whether it has a proteasome-dependent function related to the one of $HopM1_{DC3000}$. HopM1 variants are phylogenetically and functionally diverse as evidenced by the fact that a variant from *P. syringae* pv. *morspunorum* did not complement a $hopM1_{DC3000}$ deletion mutant [59]. As mentioned above, if the potential to produce conflicting virulence factors does occur in the same strain, conflict might be resolved by differential regulation of biosynthesis during host colonization.

Syrbactins in animal pathogens

The determination of SylA's structure revealed its similarity to the glidobactins (similar to cepafungins), a family of agents from soil bacteria with anti-tumor and antifungal activity isolated more than twenty years ago [62, 63]. Like SylA, glidobactin A (GlbA) contains a 12-membered macrolactam ring with its characteristic α,β-unsaturated carbonyl group (Figure 1). GlbA was shown to inhibit the ChTL and TL activities of the eukaryotic proteasome by the same mechanism as SylA, whereas the CL activity was not inhibited at all [44]. The GlbA synthetase gene cluster (*glbA-glbH*) was cloned from the soil bacterium K481-B101, which belongs to the Burkholderiales [64]. Sequence and architecture of *glbC* and *glbF*, which, like their homologs *sylD* and *sylC* in *Pss,* encode a mixed NRPS/PKS synthetase, allowed to postulate a GlbA biosynthesis model which is analogous to the one for SylA and is experimentally supported. [64, 65] (Figure 4).

Whereas disrupted nonfunctional *glb*-like gene clusters are present in strains of *Burkholderia mallei* (causing glanders in donkeys and horses), apparently intact *glb*-like gene clusters were found in the genome sequences of the human pathogens *Burkholderia pseudomallei* (the causing agent of melioidosis, β-proteobacteria), *Burkholderia oklahomensis* (β-proteobacteria), and *Photorhabdus asymbiotica* (γ-proteobacteria), In addition, an intact

glb-like gene cluster is also present in the insect pathogen and nematode symbiont *Photorhabdus luminescens*. This suggests that these pathogens have the potential to synthesize syrbactins (Figure 5) [64]. Indeed, transfer of the *glb*-like gene cluster of *Ph. luminescens* ssp. *laumondii* TT01 into *Pseudomonas putida* resulted in GlbA production in the latter species [66]. Furthermore, GlbA as well as a variant named cepafungin I, the strongest proteasome inhibitor known to date, were isolated from *Ph. luminescens* cultures [67]. Similarly, GlbA and derivatives named luminmycins have been isolated from *Ph. asymbiotica* cultures and from crickets after infection with the pathogen [68]. Thus, these pathogens indeed produce syrbactin-type proteasome inhibitors, although future research must elucidate whether and how they are involved in virulence.

Evolution of syrbactins

Syrbactin synthetase-encoding genes so far have been found in a small but diverse group of plant and animal pathogens, suggesting that they were acquired by horizontal gene transfer. Comparison of sequence and architecture of the genes encoding the NRPS/PKS synthetase modules within *syl* and *glb* gene clusters reveals that the location of the starter module genes *sylC* and *glbF* is not conserved (Figure 2 and 3). In addition, and in contrast to *sylD,* whose closest homologs found in genome databases are the *glbC*-like genes, the closest homologs of the *sylC* gene are not the *glbF*-like genes. This suggests that *sylD* and *glbC* are descendants of a common ancestral gene, whereas *sylC* and *glbF* were independently recruited into the SylA and GlbA-like synthetase gene clusters, respectively. The starter modules not only activate different amino acids, but also catalyze the addition of tails with chemically different characters [56, 65]: in the case of SylA it is an ureido-valine, leading to a hydrophilic compound, whereas in GlbA it is a fatty acid, rendering the compound much more hydrophobic (Figure 1). This difference determines whether and how syrbactins are taken up into cells. GlbA has antifungal activity [62] and evidently must be taken up by fungal cells

such as yeast, whereas yeast is completely insensitive to SylA [46]. However, yeast can be rendered sensitive towards SylA by expression of the *A. thaliana* YLS7 and YSL8 plant-specific transporters [46]. Thus the different tails may be viewed as adaptations to kingdom-specific import mechanisms.

Concluding remarks

Whereas an increasing number of T3Es in both plant and animal pathogens have been found to require the host UPS for their mode of action, proteasome inhibition as a (potential) virulence strategy has only recently become apparent in a small but interesting group of plant and animal pathogens. This group will without doubt grow in the future, but it remains to be seen how prevalent proteasome inhibition as a virulence strategy of pathogens is. In view of the involvement of the UPS in regulatory pathways controlling almost all aspects of plant life, proteasome inhibition appears, due to the expected pleiotropic consequences, rather like a drastic sledgehammer method to suppress plant immunity.This contrasts with the often exquisitely precise interference with specific host defense signal transduction components that characterizes the action of many T3Es. However, this view may be exaggerated. Tight regulation of inhibitor production [55], the inhibitor-triggered *de novo* synthesis of proteasomes as suggested by transcriptome analyses [69], as well as spacial restriction of the inhibitor to the neighborhood of infection sites due to cellular uptake and proteasome binding may render proteasome inhibition much less pleiotropic than suggested by the known involvement of the UPS in a plethora of cellular processes. This is evidenced by the fact that SylA is not a deadly toxin that kills cells, but helps biotrophic bacteria to suppress the SA-dependent defense pathway and to spread from wound infection sites [52, 53]. Further research will be required to determine the contribution of syrbactins and other proteasome inhibitors to the virulence of pathogens.

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Figure legends

Figure 1. Structure of syringolin A and glidobactin A. The α,β-unsaturated carbonyl group reacting with the N-terminal Thr of the catalytic proteasome subunits is given in red. As revealed by crystallography of the inhibitor bound to the yeast 20S CP, the double bond is opened and the C4 atom becomes linked to the oxygen in the side chain of the N-terminal Thr of the catalytic proteasome subunits, thus forming a stable ether linkage. The dipeptide bond given in green stabilizes the inhibitor upon proteasome binding, whereas the parts in blue determine active site specificity. The aliphatic tail of GlbA is shown in yellow. Adapted by permission from Macmillan Publishers Ltd: Nature [44], copyright 2008.

Figure 2. SylA biosynthesis. **(A)** Gene cluster encoding the SylA synthetase of *Pss* strains. The *sylA* gene encodes a LuxR-type transcriptional activator regulating transcription of the *sylB* gene and the *sylCDE* operon [55], whereas the *sylE* gene encodes an exporter belonging to the major facilitator superfamily. The *sylC* and *sylD* genes encode the NRPS and PKS modules, which, together with the *sylB* gene product, compose the SylA synthetase. **(B)** SylA biosynthesis model. The SylA synthetase contains three NRPS modules with condensation (C), adenylation (A), and thiolation (T) domains and a type 1 PKS module with ketosynthase (KS), acyl-transferase (AT), dehydratase (DH), ketoreductase (KR), thiolation (T), and thioesterase (TE) domains. The SylC NRPS starter module activates valine (Val) and *N*-acylates it with a second valine, thus forming an ureido linkage by incorporation of bicarbonate/CO_2 [56, 57]. The first NRPS module of SylD is thought to activate lysine (Lys), which is desaturated to 3,4-dehydrolysine by the *sylB* gene product [43]. The second SylD NRPS module is thought to activate valine, which, after condensation/decarboxylation to a malonate residue activated by the type I PKS module of SylD, results in a β-keto-thioester whose β-keto group is twofold reduced by the KR and DH domains, thus resulting in the 5-

methyl-4-amino-2-hexenoic acid (MAH) moiety. Finally, lactam bond formation between the terminal carboxyl group and the ε-amino group of the 3,4-dehydrolysine moiety catalyzed by the TE domain of the PKS module leads to cyclization and release of the final product [58]. Genes, domains/proteins and the molecular entities they act upon are given in the same color shades. Adapted with permission from [54].

Figure 3. Bayesian phylogenetic tree of 19 *P. syringae* strains. Colored bars on the left indicate phylogenetic groups as originally defined in [70]. Strains labeled in blue contain complete *syl* gene clusters. Pathovar of strain designations (if not given in the text): Pgy, *glycinea*; Pph, *phaseolicola*; Pmo, *mori*; Pae, *aesculi*; Pta, *tabaci*; Pla, *lachrymans*; Cit, unknown; Pac, *aceris*; Ppi, *pisi*; Ptt, *aptata*; Pja, *japonica*; Por, *oryzae*; Pma *maculicola*; Pan, *actinidiae*; Pmp *morsprunorum*. Figure adapted from [59] with permission.

Figure 4. GlbA biosynthesis. **(A)** The glbA synthetase gene cluster. The *glbC, glbD, and glbF* genes are shown in the same colors as their *syl* homologs in Figure 2. The *glbA* gene encodes a LysR-type transcriptional regulator [64], whereas *glbE* encodes an MbtH-like chaperone [65]. The function of the *glbB, glbG*, and *glbH* genes is currently unknown. **(B)** GlbA biosynthesis model. The GlbF NRPS starter module activates threonine (Thr) and *N*-acylates it with a coenzyme A-activated fatty acid [65]. The first NRPS module of GlbC is thought to activate lysine (Lys), which is oxidized to 4-hydroxylysine by an as yet unknown enzyme. The second NRPS module is thought to activate alanine (Ala), which, after condensation/decarboxylation to a malonate residue activated by the type I PKS module of GlbC, results in a diketide that is twofold reduced by the KR and DH domains, thus resulting in the 4-amino-2-pentenoic acid (APA) moiety. Macrolactam ring formation and release is

catalyzed by the TE domain. Genes, domains/proteins and the molecular entities they act upon are given in identical color shades. Adapted with permission from [64].

Figure 5. GlbA-like synthetase gene clusters in animal pathogens. Homolgous genes are shown in identical colors. Hatched *B. mallei* genes indicate non-functionality due to transposon (Tn) insertions and additional strain-specific mutations and rearrangements. Accession numbers of proteins encoded by functional gene clusters are as follows: K481-B101: CAL80819-CAL80826; *B. pseudomallei* K96243: YP_111277-YP_111273; *B. oklahomensis* C6786: WP_010109152-WP_010109148; *Ph. luminescens* ssp. *luminescens* TT01: NP_929149-NP_929145; *Ph. asymbiotica* ssp. *asymbiotica* (ATCC 43949): CAR67624-CAR67620. Adapted with permission from [64].

Box 1. Non-ribosomal peptide and polyketide synthesis by the thiotemplate mechanism

NRPS are large modular enzymes which synthesize peptides without the involvement of ribosomes and mRNA templates [71-73]. Each module typically contains condensation (C), adenylation (A), thiolation (T), and optional tailoring domains. Exceptions are starter modules, which normally do not contain a C domain. The A domain catalyzes the adenylation of a cognate amino acid, which becomes attached via a thioester linkage to the phosphopantethein prosthetic group of the T domain. Peptide bond formation between amino acids bound to two adjacent modules as well as release of the upstream amino acid from its T domain is catalyzed by the C domain of the downstream module. Release of the assembled product, which is often cyclized, is catalyzed by the thioeserase (TE) domain in the last module. In linear NRPS, this so-called thiotemplate mechanism leads to an assembly line in which the sequence of the modules as encoded in the genome determines the amino acid

sequence of the peptide product (e.g. as in the SylA synthetase). In non-linear NRPS, modules may act in a sequence different from the one in which they are encoded on the chromosome (e.g. as in the GlbA synthetase).

Modules of type I (modular) PKS usually contain acyltransferase (AT), ketosynthase (KS), and T domains as well as optional ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains. Attachment of coenzyme A-activated malonyl (sometimes methylmalonyl, or, in starter modules, acetyl) moieties to the T domain via a thioester bond is catalyzed by the AT domain. Condensation/decarboxylation of adjacent malonyl residues by the KS domain (which is absent in starter modules) results in a polyketide [71, 74]. β-keto groups can be reduced by the consecutive action of optional KR, DH, and ER domains, resulting in β-hydroxyl groups, α,β-unsaturated double bonds (as in SylA and GlbA), or α,β-saturated bonds, respectively. As in NRPS, the final PKS product is released, often in a cyclized form, by the TE domain of the last module. Similar to the SylA and GlbA synthetases, NRPS and PKS modules often occur together and form mixed NRPS/PKS.

Box2. Outstanding questions

- How prevalent is proteasome inhibition as a virulence strategy in pathogens?
- How widespread is the production of syrbactin-type proteasome inhibitors in plant and animal pathogens, and in microbes in general?
- What is the function of syrbactin-type proteasome inhibitors in the animal/human pathogens? Do they also enhance virulence? Could they have a function in symbiosis (e.g. in *Photorhabdus luminescens*)?

- In addition to XopJ of *X. campestris*, are other type III effectors, both within and outside the YopJ family, interfering with proteasome function?